EXHIBIT F

Endocrinology
Copyright © 1994 by The Endocrine Society

Vol. 135, No. 5
Printed in U.S.A.

# A Conserved Carboxy-Terminal Subdomain Is Important for Ligand Interpretation and Transactivation by Nuclear Receptors*

RAINER B. LANZ AND SANDRO RUSCONI

*Institut für Molekularbiologie II der Universität Zürich, 8057 Zürich, Switzerland*

ABSTRACT

Nuclear receptors share a highly conserved region located at the very carboxy-terminal part of the ligand-binding domain. Site-directed mutagenesis of conserved hydrophobic residues in this region was reported to create mouse and human glucocorticoid receptors (GRs) and estrogen receptors that cannot transactivate but apparently maintain all the other functions. We constructed analogous mutations in the rat GR to compare the mechanism of deficiency to our recently generated trans-dominant-negative mutant. We found that in the rat GR these carboxy-terminal mutations do not generate trans-dominant-negative receptors. We show that these GR mutants fail to bind dexamethasone properly, and hence receptor transformation and subsequent functions are abolished. Furthermore, we report the identification of a GR mutant that is strongly responsive to the antagonist RU486 but is silent with the agonist dexamethasone. We demonstrate that the reversal of the responsiveness is restricted to GR, since analogous mutations in related receptors do not generate similar phenotypes. Contrary to the case of the progesterone receptor, we show that carboxyl-end truncated GR mutants are not activated by RU486. We conclude that sequence conservation of this subdomain does not necessarily imply functional conservation. Chimeric constructs with GAL4 revealed the importance of protein-protein interactions to exert ligand discrimination, which is mediated by the carboxy-terminal subdomain. (*Endocrinology* 135: 2183–2195, 1994)

THE glucocorticoid receptor (GR) belongs to the nuclear receptor superfamily of ligand-dependent transcription factors. The GR and the progesterone receptor (PR), androgen receptor (AR), and mineralocorticoid receptor (MR) hormones represent a subclass of steroid receptors that bind to the same *cis*-acting sequence to regulate the responsive genes (1). These receptors are structurally and functionally related, and specific functions have been assigned to distinct domains such as ligand or DNA binding, nuclear translocation, dimerization, and transactivation of transcription (for reviews see Refs. 2, 3, 1, 4, 5). Transcription-activating functions (TAFs) have been defined both in the N-terminal domain and within the ligand-binding domain (TAF-1 and TAF-2, respectively; 6–8). For the GR, additional transactivation functions have been described within the DNA-binding region (*enh*1; 9) or in its proximity (*tau*-2; 10) (see Fig. 1B).

The mechanism of receptor activation (transformation) is complex and not as yet fully understood. Although still controversial (11), the inactive GR is located predominantly in the cytoplasm (12) in a multiprotein complex including heat shock proteins. Ligand binding then induces a conformational change that activates nuclear specific receptor functions (for review see Ref. 1). The classical view of GR transformation and functions includes binding of the hormone, dissociation of the heterocomplex, nuclear translocation (if necessary), dimerization, specific binding to DNA, and modulation of transcription.

Received April 19, 1994.

Address all correspondence and requests for reprints to: Dr. Sandro Rusconi, Institute of Biochemistry, University of Fribourg, Perolles, 1700 Fribourg, Switzerland.

* This work was supported by the Swiss National Research Foundation and by the Kanton Zürich.

The effects of antihormones have revealed ligand binding to be the key event in steroid receptor activation. Antihormones can be divided into two classes. Pure antagonists result in a total inhibition of transactivation, whereas partial antagonists possess residual agonistic activity (13). RU38486, also known as mifepristone (later referred to as RU486), was found to be an antihormone of glucocorticoids and progestins and to bind with high affinity to GR, PR, and AR (reviewed in Ref. 14). RU486 has been shown to be a partial antagonist of GR (15, 7) that promotes DNA binding of the receptor *in vivo* (16) and *in vitro* (17), whereas the same antihormone is a pure antagonist of PR (14). Hormones and antihormones might induce related, but distinct, conformational changes in the receptors. Using limited proteolytic digestion, it has been shown that transcriptional inactivation of steroid receptors by antihormones involves the induction of an inappropriate structural conformation at the extreme C-terminal end (18). An altered response to agonistic/antagonistic ligands has recently been described for a truncated PR mutant (19), supporting the importance of the conformation of the carboxyl-end of the hormone-binding domain (HBD). In this model, deletion of a region that supposedly possesses a negative regulatory function leads to a receptor mutant that can be activated by antagonists. Thus, in receptor transformation, ligand binding appears to be crucial not only for dissociation of the heterocomplex but also for modulation of the activity of the ligand-dependent transactivation domain located in the HBD.

The presence of more than one activation function makes it difficult to construct GR mutants that are unable to activate transcription while retaining all the other steroid receptor functions. In spite of that, we recently constructed a trans-dominant-negative (TDN) GR by altering the reading frame

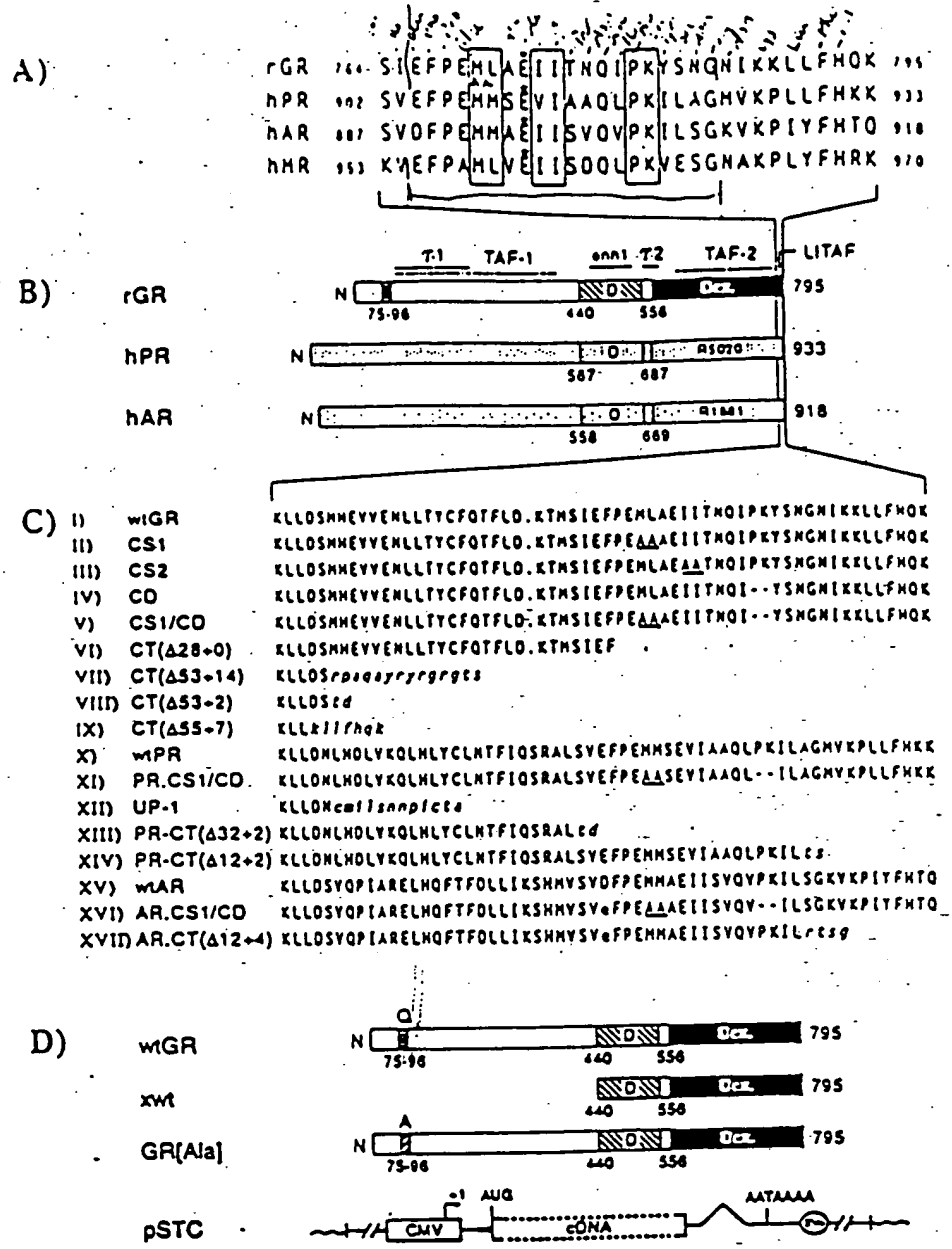


FIG. 1. Construction of receptor mutants. A, Sequence alignment of the carboxyl-end of the GR/PR/AR/MR subclass of the nuclear recept superfamily. Amino acids are shown by the *single-letter codes*. *Numbers* at *left* and *right*, positions along primary sequence. Receptors: GR (2 hPR (31), hAR (32), and hMR (38). The rGR sequence corresponds to human residues 748–777 (39). *Boxed residues*, sequences that have be mutated. In this work, the Pro-Lys residues (780/781 for rGR) will be referred to as PK-box. The Glu residue marked with an *asterisk* is conser in all the members of the nuclear receptor superfamily known so far and will be used as a position marker (Glu773 for rGR) for purposes of discussion. B, Domain structure of the rGR (*white*, *dashed*, and *filled boxes*), hPR (*heavily stippled box*), and hAR (*slightly stippled box*). T positions and extents of transactivation domains defined by different groups [TAF (7, 8), *enh* (9), and *r* (*tau*) (10)] are shown at the *top* w *dotted lines*. For rGR, the position of the poly-Gln stretch (*horizontally hatched*) in the N-terminal domain (*open box*) as well as the DBD (*dash* box) and the HBD (*filled box*, Dex) are shown. The domains of PR and AR are not distinguished by different patterning. The ligand-bind domains of the receptors are indicated by the corresponding agonists used in this work. *Numbers* indicate domain boundaries. C, Seque alignment of the carboxyl-ends of wt receptors and their mutations. Constructs are indicated with roman numerals and names at the *left*. T mutations refer to GR except where indicated by the appropriate abbreviation (PR or AR). A *dot* in the amino acid sequences of GR is a introduced for alignment to PR and AR. *Underlined* residues, substitutions of the hydrophobic residues by alanine; *dashes*, deletion of the F box; *capital letters*, natural residues; *lower-case italics*, fortuitous extensions of carboxyl-truncated mutants. UP-1, PR mutation missing 54 am acids at the C-terminal end (19); *numbers* in *brackets*, sizes of deletions followed by numbers of additional unrelated residues. D, Structure of most important GR derivatives. wtGR, full-length rGR cDNA bearing a Gln repeat between residues 75 and 96; xwt, N-terminally truncated fo lacking the major TAF-1; GR[Ala], mutant in which the Gln repeat is replaced by an alanine stretch (20; and Lanz R. B., S. Wieland, M. H and S. Rusconi, submitted). At the *bottom* is the expression vector used (pSTC, (25)); CMV, −622 to +72 promoter/enhancer region: transcription initiation; AUG, translation start site; *broken line*, cDNA insert; AATAAAA, genomic sequence of rabbit β-globin (905–2080) signals for splicing and polyadenylation; SVo, 188 base pairs of SV40 sequence spanning origin of replication; *wavy line*, bacterial plasmid (pSF including ampicillin resistance gene. Other symbols as in B.

of the CAG repeat located in the very amino terminal part of the receptor (20; and Lanz R. B., S. Wieland, M. Hug, and S. Rusoni, submitted). This mutant was called GR[Ala], since the CAG repeat is translated into a poly-Ala stretch instead of poly-Gln (see Fig. 1D). While that work was in progress, another group described substitutions in the HBD that abolish transactivation but apparently maintain all the other functions of the receptor (21), similar to our GR[Ala] mutant. The authors did not directly address the possibility that their mutants act in a dominant-negative fashion when coexpressed with wild type GR (wtGR). To compare the silencing exerted by these mutations with that of the poly-Ala stretch, we constructed the analogous mutations in the rat GR (rGR). In this paper we show that these carboxy-terminal mutants do not possess the expected dominant-negative functions. Furthermore, we report that additional mutations in the extreme carboxyl-end generate a GR that is strongly responsive to the antagonist RU486 but is silent with the agonist dexamethasone. Our data with chimeric transcription factors suggest furthermore that this response is not an intrinsic property of the mutations in the HBD alone but may require the presence of other GR domains or the action of a specific cofactor. We show that analogous mutations in the highly conserved GR/PR/AR/MR subclass do not result in parallel effects. Finally, our results provide evidence that the conserved region in the carboxy terminal part is important for ligand interpretation and may also have a role in protein-protein interaction. Hence, we propose to term this carboxy-terminal subdomain ligand interpretation and transcription activation function (LITAF).

## Materials and Methods

### *Plasmids*

The reporter constructs were based on the plasmid oligonucleotide vector (OVEC-1) (22), containing the rabbit $\beta$-globin reporter fragment −1221 to +3325 cloned into pUC18. Mammary tumor virus (MTV)-OVEC was the reporter gene plasmid for hormone responsive element activating receptors (23) and contained the mouse MTV (MMTV) promoter/enhancer (−522 to +72). 5G-OVEC, bearing five GAL4-binding sites in the enhancer position, was used as a reporter for the GAL-GR fusions (24).

The expression plasmids were all based on the eukaryotic vector pSTC (25). GR and derivatives: wtGR full-length rGR complementary DNA (cDNA) (26); GR 407-795 (xwt) and GR[Ala] were published elsewhere (20, and references therein). Carboxy-terminal substitution 1 (CS1), CS2, and carboxy-terminal deletion (CD) mutants were generated by double-stranded oligonucleotide insertions in *EcoRI–HindIII* sites of wtGR. For carboxyl-end truncation (CT) ($\Delta$28 + 0), the blunt-ended *EcoRI* site from wtGR was ligated to a *PvuII* site from the 3'-polylinker in pSCTGalX556 (25) containing stop codons in all reading frames. CT($\Delta$53 + 14) and CT($\Delta$53 + 2) truncations were generated similarly by blunt ending the *NsiI* site and ligation to *HincII* and *PvuII*, respectively. CT($\Delta$55 + 7) was constructed by excision of the 3'-*HindIII* fragment in wtGR. wtPR was generated by insertion of the *BamHI* fragment of phPR-$\beta$ (a gift of M. J. Tsai, Baylor, Houston, TX) into the pSTC vector (25), and for wtAR, the *HaeII–SalI* fragment of pSVAR0 (27) was used. The PR.CS1/CD and AR.CS1/CD mutations were generated by oligonucleotide insertions in *DraIII–BstXI* sites of wtPR and *BspI–XhoII* sites of wtAR, respectively, providing an additional *EcoRI* restriction site analogous to one present in the rGR cDNA (see below). Both PR-CT truncations were constructed by blunt ending the *DraIII* restriction site [PR-CT($\Delta$32 + 2)] or *BstXI* [PR-CT($\Delta$12 + 2)] and ligating to a *PvuII* site of the polylinker described above. In AR.CT($\Delta$12+4), XhoII cleavage was used to reconstitute the plasmid with the 3'-*BamHI* site. Sequences are shown in Fig. 1C and were verified by dideoxy chain-termination sequencing and *in vitro* translation.

All chimeric receptor constructs containing extraneous C-terminal tails were generated using the natural (GR) or engineered (PR and AR; see above) *EcoRI* restriction site. For PR-GR(HBD) chimeras, the receptors were swapped at the corresponding *NspHI/SphI* sites located in the second zinc finger-coding part. For the GR-AR(HBD) constructs, the AR cDNA restricted with *HpaII* was fused to the *PstI* site 3' to the DNA-binding domain (DBD) of GR using a short (*AccI/PstI*) polylinker segment of pSP64, yielding an additional glycine residue. GAL4-Act/GR(HBD) constructs are based on pSCTEV (24), containing the DBD of GAL4 (amino acid residues 1–93) from pSG424 (28) and the GAL4 activator region II fragment (*HindIII–SmaI*) from pSCTGalX556 (25). The GR segments from wtGR, CS1, CS2, CD, and CS1/CD were fused to the 3'-*SmaI* site of pSCTGalX556 by blunt ending the GR fragments restricted with *AccI* at amino acid position 500 using the mutagenesis cassette described elsewhere (29).

### *Cell culture and transfection assays*

CV-1 (American Type Culture Collection, Rockville, MD) cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum and penicillin/streptomycin. Cells were transfected by the calcium phosphate coprecipitation procedure (22, and references therein) with 10 μg reporter plasmid, 0.1–5 μg transactivator plasmid, 3 μg reference plasmid (22; see also Fig. 2), 8–10 μg sonicated salmon sperm DNA as carrier, and a mock expression plasmid, cytomegalovirus promoter-chloramphenicol acetyltransferase (CMV-CAT), used in negative controls to compensate for different amounts of CMV-bearing plasmids.

Where indicated, $5 \times 10^{-7}$ M dexamethasone (Dex), $1 \times 10^{-6}$ M mifepristone (RU486), $5 \times 10^{-8}$ M promegestone (R5020), or $5 \times 10^{-7}$ M methyltrienolone (R1881) was present throughout the incubation. Transiently expressed RNA was isolated 48 h after rinsing and subjected to S1-nuclease analysis (22). Quantification of the signals was performed by laser densitometric scanning of different film exposures. The reporter signal (Sig in Fig. 2) and the reference signal (Ref in Fig. 2) give distinct bands in this assay. The corrected transcription is defined as the ratio (signal:reference, see Fig. 2 legend). For clarity, some standard samples (see Fig. 2) are considered as 100%, and the relative transcription is then defined by comparing individual corrected transcription with these standards (see *bottoms* of panels in Fig. 2 and *numbers* at *right* in Fig. 4).

For the *in situ* immunofluorescence experiments (Table 1), 10 μg effector plasmid carrying the mutated rGR cDNA and an SV40 origin of replication were transfected together with 1 μg of an expression vector encoding SV40 T-antigen (25, 29). Cells were incubated for 24 h after removal of the $CaPO_4$ precipitate. Hormone was added 2 h before fixation. The cells were fixed with acetone:methanol (3:7) at −20 C, dried and treated with a monoclonal antibody (BUGR; 17, 30). The complexes were visualized with fluorescein isothiocyanate-labeled goat antirabbit antibody (Calbiochem, La Jolla, CA).

### *cRNA synthesis and in vitro translation*

For *in vitro* transcribed and translated recombinant cDNAs, the N terminally truncated derivatives (xwt, xCS1, *etc.*) were used. The mutants were subcloned into the plasmid pSCTX795 (25), linearized by *BglII* restriction, transcribed with T7 RNA polymerase and messenger RNA translated in a rabbit reticulocyte lysate system as recommended by the supplier (Promega Biotec, Southampton, UK).

### *Ligand-binding assays*

The filter binding assay and the dexamethasone-mesylate binding assay have been described previously (17). Relevant details are given in Figure 3. [$^3$H]Dex was from Amersham (Buckinghamshire, UK). [$^3$H]Dex-mesylate from DuPont-New England Nuclear (Boston, MA), and unlabeled and labeled RU486 (mifepristone) were gifts from Roussel Uclaf (Romainville, France).

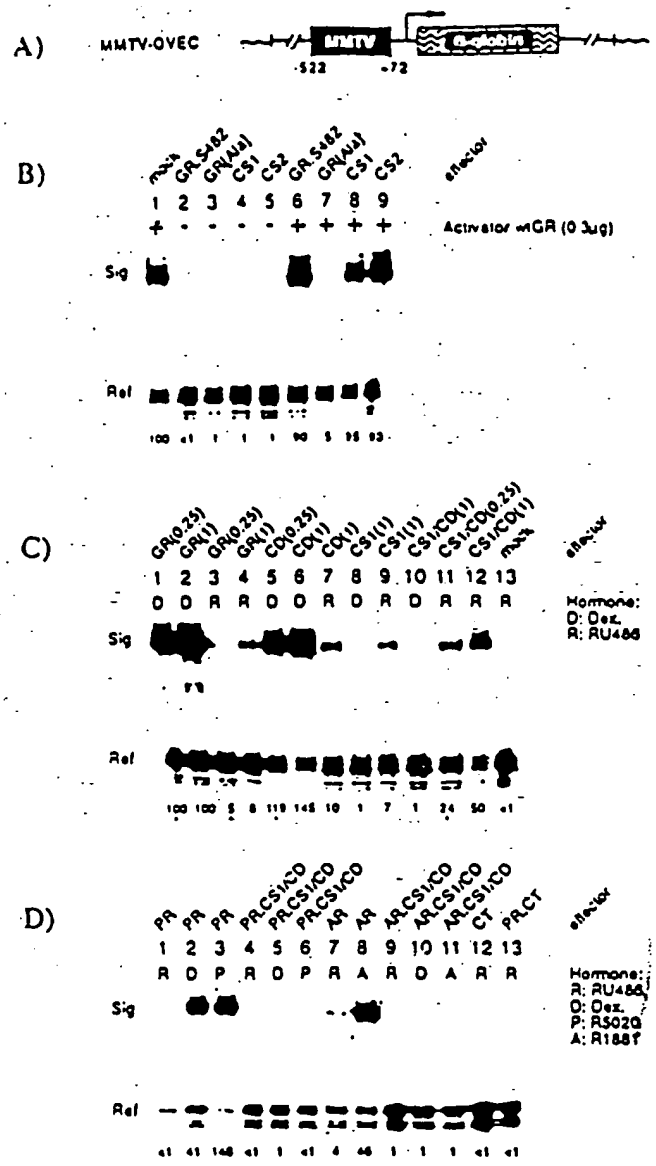


FIG. 2. Transactivation properties of GR mutants. A, MMTV-OVEC is the reporter gene plasmid containing the MMTV promoter/enhancer (reviewed in Ref. 40) linked to the rabbit β-globin reporter fragment (22). Transient transfection experiments were done in CV-1 cells by calcium phosphate coprecipitation of typically 0.3–3 μg effector plasmids, 10 μg reporter vector, 3 μg reference plasmid (22), and carrier DNA to 25 μg. The expression of β-globin-RNA was analyzed by quantitative S1-nuclease mapping (see *Materials and Methods*; 22). The panels show representative autoradiograms in which both the test signal (Sig) and the reference signal (Ref) are visible. Relative transcription was determined densitometrically (see *Materials and Methods*) and is presented at the *bottoms* of radiograms; standard samples are defined as 100% and deviation is less than 5%. D, $5 \times 10^{-7}$ M Dex; R, $5 \times 10^{-9}$ M RU486; P, $5 \times 10^{-9}$ M progestin R5020; A, $5 \times 10^{-7}$ M androgen R1881. B, Transrepression properties of the carboxy-terminal GR mutants in comparison with the GR[Ala] mutant. Names of effector plasmids are shown at the *top*; +, 0.3 μg plasmid expressing wtGR. Lanes 2 to 5 show transactivity of 3 μg expression vector encoding GR mutants alone; lanes 6 to 9 demonstrate the ability to repress the action of 0.3 μg coexpressed vector encoding wtGR. The corrected transcription level in lane 1 is defined as 100%. Lane 1, Activity of 0.3 μg pSTCwtGR together with 3 μg inert effector plasmid CMV-CAT (mock); lane 2, negative control pSTC GR(S482) with destroyed second zinc finger structure (29). GR[Ala] (lane 3) as well as CS1 and CS2 (lanes 4 and 5) do not transactivate, but only GR[Ala] is able to repress the action of coexpressed wtGR (lane 7). Hence, the carboxy-terminal substitution mutants CS1 and CS2 are not TDN. C, Transactivation properties of the C-terminal GR mutants in the presence of agonist (Dex) and antagonist (RU486). *Names* and *bracketed numbers* at *top* of panel indicate type and amount (in micrograms) of effector plasmid; other symbols and transfection conditions as described above; control, 3 μg inert effector plasmid CMV-CAT (lane 13, mock). The activity derived from 0.25 μg (*asterisks* labeled value, lane 1) and 1 μg (lane 2) pSTCwtGR in the presence of Dex are individually defined as 100%. The double mutant CS1/CD is not responding to the agonist (lane 10) but shows an increased response to the antagonist RU486 (lane 12) compared with the wtGR (lane 4). D, Transactivation properties of the carboxy-terminal mutations in PR and AR. *Names* at *top* of panel, type of effector plasmid (1 μg throughout); other symbols and transfection conditions as described above. Lane 12, CT is GR-CT(Δ53+14); lane 13, PR-CT (Δ32+2). The corrected transcription observed by cotransfection of 1 μg pSTCwtGR in the presence of Dex (lane 2 in C) is defined as 100%. The CS1/CD mutation demonstrates the RU hyperreaction only in the context of GR.

## Results

### *Substitution of two hydrophobic residues by alanine in the TAF2 of the receptor does not generate dominant-negative mutants*

By changing the translational reading frame of the CAG repeat toward the amino-terminal coding portion of the rGR cDNA, we have previously generated a mutant that is incapable of transactivation but maintains competence for hormone binding, nuclear translocation, and specific DNA binding (20; Lanz R. B., S. Wieland, M. Hug, and S. Rusconi, submitted). This functional variant (which is called GR[Ala], since the CAG repeat is translated into an alanine stretch; see Fig. 1D) displays negative dominance when tested for transcriptional activation *in vivo* (see Ref. 20 and below).

A recent report described amino acid substitutions at the C-terminus in mouse GR (M758A/L759A) which, in analogy to our GR[Ala] mutant, allegedly turned off the transactivation potential of the receptor while maintaining the other functions intact (21). We wanted to examine whether these mutants also behave as TDN GRs. To this purpose we constructed the equivalent CSs in the rGR (M770A/L771A = CS1 and I774A/I775A = CS2; see Fig. 1, constructs II and III). We have cotransfected effector plasmids encoding the mutant GRs along with a reporter vector in which the rabbit β-globin gene is driven by the MMTV promoter (MTV-OVEC; Ref. 23; Fig. 2A). The level of reporter messenger RNA was analyzed by quantitative S1-nuclease protection, and representative results are shown in Fig. 2, B–D. Small amounts (0.3 μg) of effector plasmid bearing the wtGR stimulate the reporter gene in the presence of Dex (Fig. 2B, lane 1), whereas large amounts (3 μg) of the CS1- or CS2-encoding plasmids are completely inactive, in agreement with previous findings (21; Fig. 2B, lanes 4 and 5).

To test whether the GR carboxy-terminal mutants CS1 and CS2 can act as TDNs, we expressed a given amount of wtGR and challenged its action by coexpression of the mutants. As shown in Fig. 2B, neither CS1 nor CS2 is able to compete with the wtGR for the GR-responsive MMTV long terminal repeat promoter (see Fig. 2B, lanes 8 and 9, for

TABLE 1. Properties of carboxy-terminal receptor mutants

| Construct[a] | Functions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ligand binding[b] | | | Localization[c] | | | Transaction[d] | | |
| | | | | | | | Repr | Activation | |
| | Dex | RU | Mes | None | Dex | RU | Dex | Dex | RU |
| 1 wtGR | +++ | +++ | +++ | N > C | N | N | • | ++++ | – |
| 2 GR[Ala] | – | +++ | +++ | C > N | N | N | yes | – | – |
| 3 CS1 | – | +++ | +++ | N = C | N > C | N | no | – | (–) |
| 4 CS2 | – | +++ | +++ | N = C | N > C | N | no | – | (–) |
| 5 CD | +++ | +++ | +++ | N > C | N | N | • | ++++ | + |
| 6 CS1/CD | – | +++ | +++ | N = C | N > C | N | • | – | +++ |
| 7 CT | | + | | C > N | C > N | N | | – | – |
| 8 GAL4-Act/GR(HBD) | | | | C | C > N | C > N | | + | (+) |
| 9 GAL4-Act/GR(HBD).CS1/CD | | | | C | C > N | C > N | | – | (+) |

The table semiquantitatively displays the receptor functions. Data shown in Figs. 2–4 are included. General structure and details on sequence of the constructs are given in Figs. 1 and 4. Empty fields indicate experiments that have not been done or are not relevant for the points addresse in this work.

[a] Construct names are indicated at the left and refer to GR.

[b] Ligand-binding data was obtained with receptor derivatives synthesized *in vitro* and by filter binding [Dex and RU486 (RU)] or s electrophoresis of immunoprecipitated receptors [Dex-Mesylate (Mes)] (17) (Fig. 3): amino-terminally truncated receptors were used. ++ Ligand-binding indistinguishable from or better than wild type; +, clearly reduced affinity; –, close to background.

[c] Cellular localization was determined by *in situ* immunofluorescence in absence (none) or in the presence of the agonist Dex and the antihormo RU486 (RU). N, Nucleus; C, cytoplasm.

[d] Transaction: the ability to transrepress (repr.) or to transactivate (activation) is displayed qualitatively. Transactivation data were obtain by transient transfection of effector and reporter plasmids and analyzed by S1-nuclease mapping. The results include the radiograms represent in Fig. 2. Transrepression refers to the ability of the mutant to repress the signal derived from co-transfected wtGR (20; Lanz R. B., S. Wielar M. Hug, and S. Rusconi, submitted). •, Not determinable; no and yes, unable, respectively, able to transrepress; ++++, transactivati indistinguishable from—or stronger than—wild type; +++, 40–60%; +, 5–10%; (+), 1–5%; –, close to background (<1%).

competition in 10-fold plasmid excess), whereas under similar conditions our GR[Ala] mutant acts as a strong repressor (Fig. 2B, compare lanes 3 and 7). Hence, the mechanism of GR deactivation in CS1 and CS2 mutants must be different from the one operating in the GR[Ala] mutant. We reasoned that CS1 and CS2 mutants might have lost more than just their transactivation properties, and we proceeded by examining their ability to bind hormones and to react to antagonists. In the course of CS1 and CS2 mutagenesis, some additional mutants arose, and they were tested in parallel.

### *Mutations that increase responsiveness to the antagonist RU486*

In parallel to the CS1 and CS2 mutations, we focused our interest on one spontaneous variation obtained during cloning. This mutant harbors, in addition to the hydrophobic substitution, also a deletion of two amino acids (Pro 780 and Lys 781, referred to as PK-box in this work) located seven residues C-terminally from an invariant glutamic acid (position 773 in rGR; see Fig. 1A). The double mutant GR that includes both the Ala substitutions A770/A771 (CS1) and the CD 780–781 has been called CS1/CD (see Fig. 1C, construct V).

Since it has been shown that hormone and antihormone induce distinct conformational changes that are central to steroid receptor activation (18), we considered it worthwhile to also test all the carboxy-terminal mutations in the presence of the GR antagonist RU486 (Fig. 2C). In our assays, the average of agonistic activity of RU486 on the wtGR is approximately 10% relative to full activation by Dex (Fig. 2C, lanes 3 and 4). We observed that the mutant CS1 retains the ability to partially respond to RU486 (lane 9). More intere ingly, we observed that the transactivation by the CS1/( mutant in the presence of RU486 is remarkably strong (la 11 and 12). The double mutant shows an RU486-induc transactivation that reaches about 50% of that of the wt in the presence of Dex (Fig. 2C, compare lane 12 with 2 11 with 3). Consistent results were obtained by varying ligand concentrations ranging from 5 nM–1 μM RU486 by changing the amount of effector plasmid in transfect assays (data not shown). We could exclude that the enhan response to RU486 is due to spurious mutations in original construct by reimplanting a small entirely sequer segment containing the mutated region in the original cDNA (not shown).

Since the mutant CS1 maintains partial transactivatio the presence of RU486 (Fig. 2C, compare lane 4 with 9) wanted to test whether the increased response of the ( CD mutant is solely due to the deletion of the PK-box. He we separated the mutations (see Fig. 1C, construct IV, and tested them again in the presence of different lig Transient transfection of various amounts of plasmid pressing wtGR or CD in CV-1 cells results in compa stimulation of the GR-responsive MMTV long termin peat promoter in the presence of Dex (Fig. 2C, compare 1 and 2 with 5 and 6). When the PK-box deletion m (CD) was assayed for the response to RU486, it disp the original partial agonistic effect of wtGR (compare 1 with 7). This demonstrates that the deletion of the P does not alter the transactivation properties of the rec *per se*. Rather, we have to conclude that the increased R response in the CS1/CD double mutant is the result

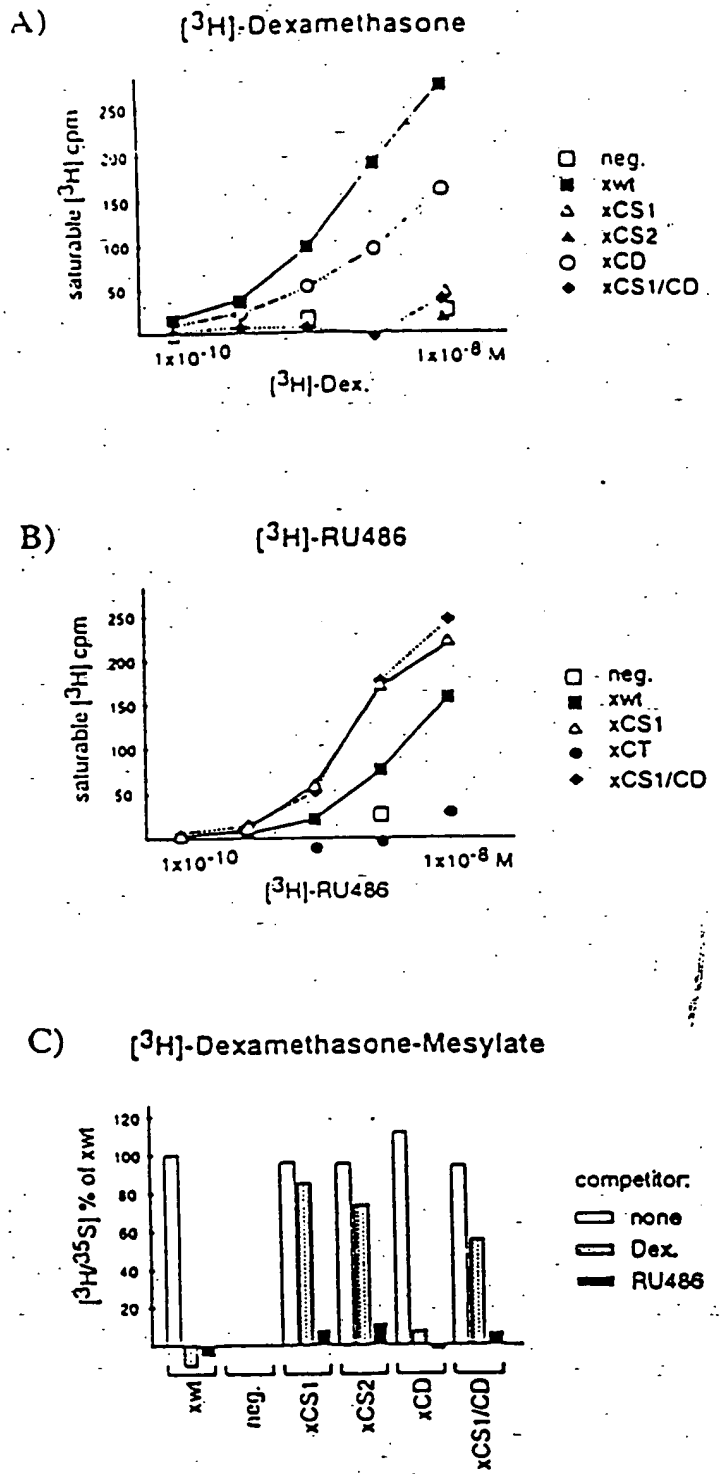


FIG. 3. Ligand-binding properties of GR derivatives synthesized *in vitro*. A and B, Dex and RU486 equilibrium labeling. *In vitro* translation reactions were carried out in the presence of various concentrations of [$^3$H]Dex (A) and [$^3$H]RU486 (B), respectively, and receptor binding was assayed by filter binding (17). The GR derivatives are truncated versions lacking the N-terminal parts (see Fig. 1D), indicated by x. Y-axis, Saturable $^3$H-labeled counts per min per filter (see *Materials and Methods*); X-axis, concentrations of $^3$H-labeled hormone. *Open squares*, neg, negative control GRx556; *filled squares*, xwt; *open triangles*, xCS1; *filled triangles*, xCS2; *open circles*, xCD; *filled circles*, xCT; *filled diamonds*, xCS1/CD. The labeling reactions reveal that the substitution mutants xCS1, xCS2, and xCS1/CD have practically lost Dex-binding property (A) but retain the ability to bind the antihormone RU486 (B). C, [$^3$H]Dex mesylate affinity labeling. Unlabeled *in vitro* translation reaction products were incubated with 0.2 μM [$^3$H]Dex mesylate in the presence or in absence of 10 μM unlabeled competitor. Receptor derivatives were immunoprecipitated, electrophoresed, and analyzed by radiography. Specific signals were evaluated densitometrically (see *Materials and Methods*). The graph represents the relative amounts of [$^3$H]Dex mesylate-labeled receptor. The signal calculated from xwt is defined as 100%. Y-axis, Relative [$^3$H]Dex mesylate labeling; X-(category) axis, receptor mutants. *Open bars*, no competitor in the binding reaction; *stippled bars*, 10 μM unlabeled Dex as competitor; *filled bars*, 10 μM unlabeled RU486 as competitor. The affinity labeling shows that the carboxy-terminal mutants still can bind Dex mesylate. The labeling of the CS1, CS2, or CS1/CD mutants cannot be competed by Dex.

combined action of the mutation CS1 (substitution of two hydrophobic amino acids, positions 770 and 771 in rGR by alanines) together with the deletion CD (elimination of the PK-box). Table 1 summarizes further properties of the CD mutant, which will be discussed later. A combination of the mutants CS2 and CD was not constructed.

*The effects of the CS1/CD double mutation are GR specific*

A number of functions described for receptors of the GR/PR/AR/MR subclass have been shown to be shared by the other members. To verify whether the increased RU486 responsiveness can be transferred to another receptor, we constructed the point mutations corresponding to CS1/CD in the human PR (hPR; 31) and hAR (32) (see Fig. 1B, PR.CS1/CD and AR.CS1/CD, constructs XI and XVI). These variants were tested for transactivation in the presence of agonist and antagonist. In our assay, the wild type hPR demonstrated the expected activation in the presence of the progestin agonist R5020 (Fig. 2D, lane 3) and null response toward RU486 (lane 1). We observed that PR was responding significantly to Dex (lane 2), an observation that is consistent with the partial *in vitro* PR activation observed by others (33). Contrary to our expectation, the PR.CS1/CD mutant was responding differently than the GR-CS1/CD, since it did not show an enhanced response to the antagonist RU486 (Fig. 2D, lane 4). This double mutant cannot transactivate when induced with the synthetic glucocorticoid Dex or with the synthetic progestin R5020 (Fig. 2D, lanes 5 and 6). Similarly, the corresponding mutations in the hAR demonstrated a lack of transactivation in response to RU486, Dex, and the synthetic androgen R1881 (Fig. 2D, lanes 9–11). Hence, the exhibition of the altered hormone responsiveness by the GR cannot be simply transferred to hPR or hAR by generating corresponding mutations. We conclude that although the mutations affect very conserved amino acids, the functional significance of the residues must be different for each receptor.

*Unlike PR, carboxyl-end truncated GR mutants are not activated by RU486*

After observing that an effect generated in the GR cannot be transferred to PR or AR, we tried to verify whether a

property found in a PR mutant can be transferred to GR. An inversion of the response to agonistic/antagonistic ligands has recently been described for a truncated PR mutant called UP-1 (19) that is missing the C-terminal 54 amino acids (see Fig. 1C, construct XII). Reportedly, this mutant can bind neither progesterone nor the synthetic agonist R5020 but can still bind RU486 and strongly activate transcription in the presence of this antagonist. The authors interpreted these data by arguing that the most C-terminal portion of the receptor might contain an inhibitory function that silences receptor transactivation activity in the absence of the agonist and is not displaced in the presence of the antagonist. They also suggested that the inhibitory effect of the C-terminal tail on the transcriptional potential of antagonist-bound receptor might be a general phenomenon for steroid receptors. In our hands, the equivalent truncation of the very C-terminal part in rGR does not result in a mutant receptor responding to RU486. Using available restriction sites in GR cDNA, we generated different-truncation mutants (generally referred to as CTs: Δ28 + 0, Δ53 + 14, Δ53 + 2, and Δ55 + 7, where the numbers describe the sizes of the deletions followed by the amounts of additional unrelated residues; Fig. 1C, constructs VI–IX). We also used the truncated mutant N-766, whose properties have already been published (17). All these truncated GR mutants were completely silent in transactivation in the presence of both the agonist Dex and the antagonist RU486. In Fig. 2D, lane 12, we show the lack of transactivation of one representative of the GR CT, namely Δ53 + 14, which is the closest analog of the reported PR mutant in terms of position of the truncation as well as lengths of the unrelated amino acid residues (Fig. 1C, compare constructs VII and XII).

By using given restriction sites in the PR cDNA, we constructed two CT PR derivatives (Fig. 1C, constructs XIII and XIV) and tested them for transactivation. Neither demonstrated any activation in the presence of either the agonist R5020 or the antagonist RU486 (Fig. 2D, lane 13 for the response to RU486). We did not reconstruct the published UP-1 mutant. The different transactivation could be the effect of the additional unrelated amino acids or the different position of truncation between the published PR mutant and our GR CTs and PR derivatives. A truncated AR mutant, AR:CT (Fig. 1, construct XVII), missing only a few amino acids at the C-terminus, cannot transactivate in the presence of R1881, Dex, or RU486 (see Table 1).

*Altered affinity for the ligand in the C-terminal GR mutants*

To analyze the ligand-binding ability of the mutants, we performed equilibrium labeling experiments with *in vitro*-generated receptor derivatives. It has been shown that the N-terminally truncated receptor x795 is indistinguishable in its hormone-binding capacity from the intact receptor N795 (17). Furthermore, *in vitro* translation of x795 constructs gives higher yields than full-length constructs (our unpublished results). Hence, for the *in vitro* experiments, we used truncated versions lacking the N-terminal domain (see xwt in Fig. 1D; the corresponding carboxy-terminal mutants are xCS1, xCS2, xCS1/CS, and xCT). The recombinant plasmids carrying the DNA- and the hormone-binding portions of the receptor-coding region were transcribed and translated *in vitro* (see Ref. 17 and *Materials and Methods*). The GR proteins were synthesized in the presence of various concentrations of [$^3$H]Dex or [$^3$H]RU486, and the ligand binding was measured using a filter binding assay (17). Figure 3A shows the equilibrium labeling of some GR mutants in the presence of [$^3$H]Dex, varying from 1 nM to approximately 0.1 μM. The *curves* represent the amount of saturable high affinity sites. In accordance with the transactivation experiments, the xCD mutant (Fig. 3A, *circles*) was similar to xwt (Fig. 3A, *filled squares*) for labeling [$^3$H]Dex, whereas xCS1/CD (Fig. 3A, *filled diamonds*) remained close to the background (compare it with the negative control x556, Fig. 3A, *open squares*). Contrary to the results reported by others (21), in this approach neither xCS1 nor the xCS2 GR mutations display significant [$^3$H]Dex binding. Thus, although the apparent equilibrium dissociation constant of the deletion mutant xCD is not severely altered, the xCS1/CD or the xCS1 and xCS2 mutants have virtually lost their Dex-binding property. Analogous equilibrium-labeling experiments were done using [$^3$H]RU486. Both the Ala-substituted xCS1 (Fig. 3B, *open triangles*) and xCS2 mutants and the double mutant xCS1/CD (Fig. 3B, *filled diamonds*) demonstrated labeling with [$^3$H]RU486 even stronger than xwt (Fig. 3B). The antihormone bound with comparable affinity to the deletion mutant xCD and xwt, whereas binding to the C-terminally truncated mutant xCT was barely detectable (Table 1). Taken together, the results obtained by equilibrium labeling with [$^3$H]Dex and [$^3$H]RU486 demonstrate that the analyzed GR point mutations do not dramatically alter the binding of the antihormone RU486. However, Dex binding is significantly reduced in the substitution mutants, indicating that the hydrophobic residues Met/Leu preceding the conserved Glu77 (Fig. 1A) are important for retention of Dex but not RU486.

We also examined the binding of the mutated receptors to [$^3$H]Dex mesylate. This electrophilic affinity-labeling derivative of Dex associates covalently with cysteine residues (3). *In vitro* translation reaction products were incubated in the presence of 0.2 μM [$^3$H]Dex mesylate. A portion of each labeling reaction also contained excess (10 μM) unlabeled Dex or (1 μM) RU486, respectively, which compete only for the saturable, high affinity sites of the receptor. After binding the labeled proteins were immunoprecipitated, fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and analyzed by radiography. The signals have been quantified in Fig. 3C. The graph shows that all the mutants except the negative control x556 can bind [$^3$H]Dex mesylate (*open bars*). In this assay the xCS1, xCS2, and xCS1/CS mutants were indistinguishable from xwt reactivity for the ligand, whereas xCD was slightly stronger. The affinities of some of the carboxyl-end truncated mutants xCT (x766 and x742 + 14) for [$^3$H]Dex mesylate were determined to be less than 10% relative to the binding of xwt (not shown). The affinity label is not significantly competed by a large excess of cold Dex in the reactions including xCS1/CD, xCS1, and xCS2 (Fig. 3C, *stippled bars*) but is competed in reactions containing xCD or xwt. This indicates that mutation of

paired hydrophobic amino acids (Met-Leu and Ile-Ile, respectively) strongly influences Dex binding without affecting the capacity to bind Dex mesylate. In the presence of RU486 as competor, all the tested samples lost the labeling by [$^3$H] Dex mesylate (Fig. 3C, *filled bars*). The affinity-labeling experiment clearly demonstrated that the carboxy-terminal GR mutants can still strongly bind [$^3$H]Dex mesylate, but that competition by Dex is altered. Therefore, if we assume that Dex mesylate, Dex, and RU486 have similar on rates, we have to conclude that the off rate of the agonist Dex (but not of the antagonist RU486) is probably significantly affected in these mutants.

*Other domains of the mutated receptor may participate in the altered hormone response of the CS1/CD mutant*

We could show that transplantation of the CS1/CD mutations in PR and AR does not result in an increased response to RU486 (Fig. 2D and Fig. 4, lines 11 and 16) and argue that the mutations *per se* are not sufficient for the abnormal behavior in GR. We constructed further chimeric proteins by progressive replacement of the GR-CS1/CD mutant with analogous parts of either PR or AR (Fig. 4). In the fusion to PR, only proteins containing the very C-terminal portion of GR-CS1/CD maintained elevated responses to RU486 (Fig. 4, compare lines 5 and 6 and 7 and 8), whereas chimeras with C-terminal PR mutated portions maintained the pure antagonistic response to RU486 (Fig. 4, lines 11–14). Interestingly, the fusion proteins with mutated PR carboxyl-ends (PR.CS1/CD) cannot transactivate in the presence of the R5020 (Fig. 4, lines 11, 13, 14, and 18). None of the fusion proteins to AR exhibits increased responsiveness to RU486, even if the very C-terminal tail is derived from GR-CS1/CD (Fig. 4, lines 15–20). An exception is the chimera PR/AR-CS1/CD, in which the C-terminal tail of PR is replaced by the mutated counterpart of AR. This construct also showed an increased response to RU486 (Fig. 4, line 9). We conclude that the very C-terminal tail of the mutated GR is not sufficient for the abnormal response to RU486. All the fusion proteins containing the CS1/CD mutations failed to be activated by Dex.

For GR, the weak agonistic activity of RU486 is supposed to be due to the hormone-independent TAF-1 (16). We deleted this domain in wtGR and noticed that the amino terminal truncated derivative xwt has a partial response to RU486 of about 1.5% relative to full-length receptor after Dex induction (Fig. 4, line 24). The corresponding truncation in the CS1/CD mutant does not respond as strongly as the full-length version to RU486 (line 25), although this version also shows a clearly elevated response to the antagonist. This mutant is still unable to respond to the agonist. We suspected therefore that other domains of the receptor might be involved in the altered antagonist response by the CS1/CD mutant. To measure the autonomy of the HBD in the increased activity to RU486, we constructed fusion proteins with the yeast transcription factor GAL4 (see Fig. 4, constructs 26–30) and tested them for transactivation on reporters bearing GAL4-binding sites. Transcription from the reporter was rather low in these transfection experiments. Mobility shift assays with nuclear extracts and *in situ* immunofluorescence analysis revealed a hindered nuclear translocation of the GAL4-fusion proteins (Table 1). Nevertheless, the GAL4 fusion containing the wtGR responds well to Dex induction, whereas the partial agonist effect of RU486 is almost insignificant (Fig. 4, line 27). The chimera containing the HBD of CS1/CD does not show an increased response to RU486 (line 28). The GAL4-fusion constructs with the separated mutations (CD, CS1, and CS2, respectively) demonstrate parallel transactivation to the one observed in the GR context (Fig. 4, compare lines 29 and 30 with 3 and 2). Since the chimeric GAL4 constructs do not contain the GR zinc finger and *tau*-2 region, we conclude that the activity of either one of these could be involved in the increased responsiveness of the CS1/CD mutant to RU486.

## Discussion

We have shown elsewhere that alternative translation of the naturally occurring CAG repeat into a polyalanine stretch completely silences transactivation of the rGR without affecting the other receptor functions (20; Lanz, R. B., S Wieland, M. Hug, and S. Rusconi, submitted). This GR[Ala] can act as a transrepressor over wtGR and other related receptors such as PR and AR. Others reported point mutations in a conserved region in the HBD of GR and estrogen receptor (ER) that significantly reduced ligand-dependent transactivation but had no effect on steroid or DNA binding (21).

The original aim of this work was to establish whether these mutants would act as TDNs similar to GR[Ala]. We therefore constructed analogous mutations in rGR and analyzed receptor functions. Our results can be summarized as follows: 1) replacement of either pair of hydrophobic residues flanking the conserved glutamic acid by alanines strongly reduced hormone binding, in contradiction with the original claims (21); consequently, these mutants are not TDN; 2) a mutant bearing both the substitution and a fortuitous deletion of the PK-box was able to strongly activate transcription in the presence of the antagonist RU486 but was silent on Dex induction; 3) the PK box deletion *per se* does not alter the transactivation properties of the GR; 4) corresponding mutations in PR and AR did not show the increased response to RU486 as exerted by the GR mutant; 5) unlike reports on PR, carboxyl-end truncated GR mutants are not activated by RU486; and 6) GAL4-fusion proteins lost the enhanced RU486 response. Our results strongly indicate the importance of the conserved region in the carboxyl-end of the receptor in ligand discrimination and in distinction of agonistic from antagonistic modulation and suggest that this region works in concert with other receptor domains.

The HBD of nuclear receptors encompasses several distinct receptor functions, which cannot be attributed to specific regions of these receptors (for review see Ref. 4). Ligand binding appears to involve finely scattered elements of the large domain, since most of the mutations identified in the HBD compromise the ability of the mutated receptor to bind hormones (see reviews, Refs. 1, 4, 5). Similarly, large sections of this domain are required to provide the interacting sites for receptor-associated proteins, which silence receptor activ-

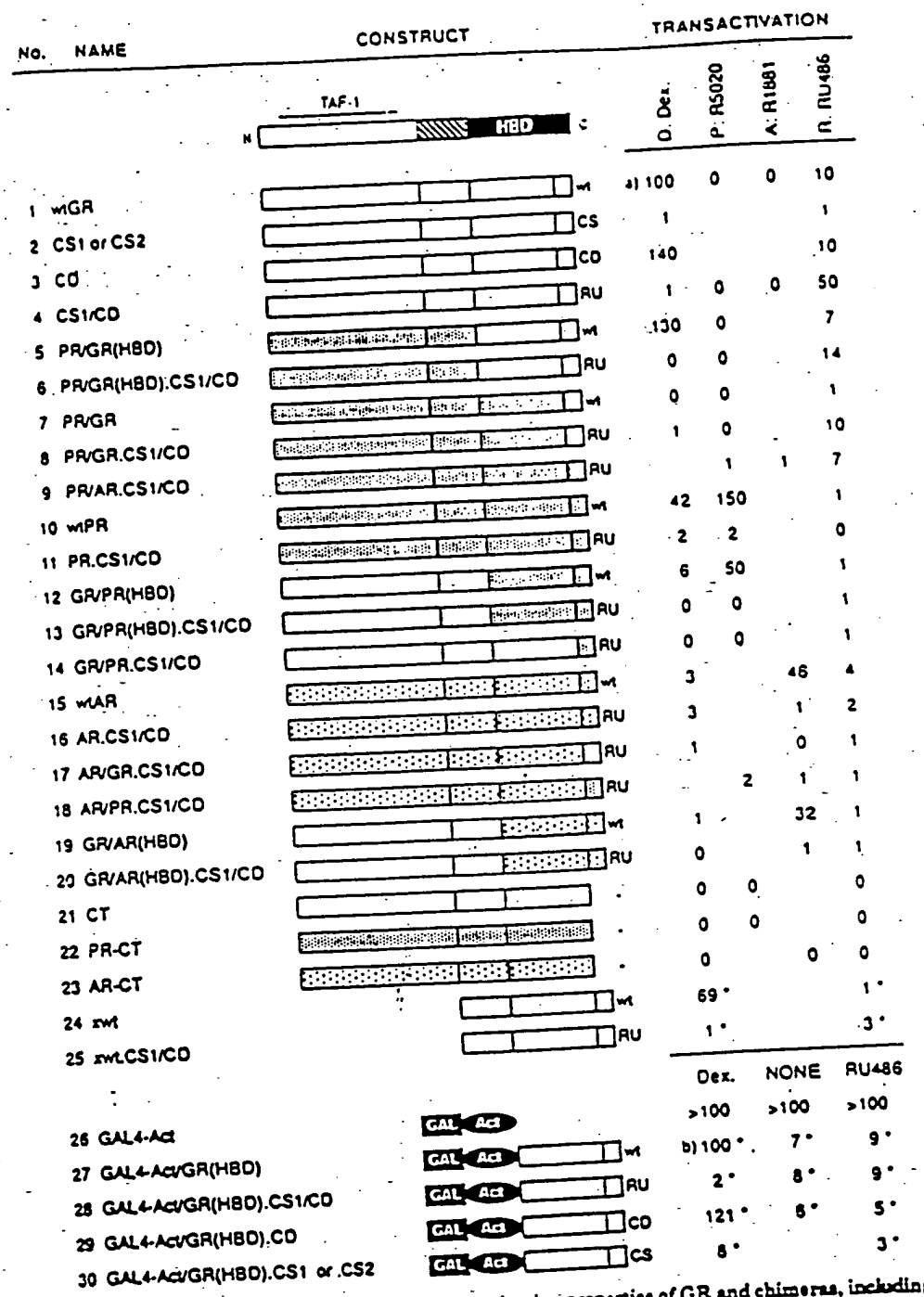


FIG. 4. Transactivation properties of GR mutants and chimeras. Transactivation properties of GR and chimeras, including some of those descri above. The constructs are indicated by *numbers*, *names*, and by a schematic illustration with sections representing the domain boundaries (un Fig. 1, here not in scale). Construct names refer to GR, except where indicated by the corresponding receptor abbreviation. x, Amino-trunc GRs (Fig. 1D); GAL, DBD of the yeast transcription factor GAL4; Act, activation region 2 of GAL4 (41). The C-terminal box is part of the H and illustrates the portion downstream of the *EcoRI* site in rGR cDNA (residues EF768/767–795). This segment includes the carboxy-term mutations reported in this work. The *EcoRI* site was created at homologous positions in AR and PR and used for swapping the portion chimeras. *Open rectangles*, Domains derived from the GR; *heavily stippled areas*, sections from PR; *lightly stippled areas*, portions from Abbreviations at the *right* of the C-terminal box indicate the status of the sequence (see Fig. 1C). RU, Double mutation CS1/CD. CTs are displ as if missing the entire boxes, although the termination is not precisely at the *EcoRI* site (see Fig. 1C and *Materials and Methods*). transactivation capacities of the mutants were determined by transient transfection experiments and analyzed by S1-nuclease mapping as descr in Fig. 2. Constructs 1–25 were tested for transactivation on MMTV-OVEC (Fig. 2A), constructs 26–30 on 5-fold GAL4-OVEC (24). The displ relative transactivations are average values derived from several independent experiments. For constructs 1–25, the activities in the presen Dex ($5 \times 10^{-7}$ M), R5020 ($5 \times 10^{-8}$ M), R1881 ($5 \times 10^{-7}$ M), and/or RU486 ($5 \times 10^{-8}$ M) are relative to the activity of wtGR on Dex induction For lines 26–30, the activity obtained with the intact HBD of wtGR fused to GAL4-Act (construct 27) is defined as 100% (b). The *asterisk* indi that particular constructs were transfected in higher amounts (10-fold) to compensate for the lacking of the major TAF-1 (constructs 24 an or for the poor nuclear localization (constructs 27–30).

ity in the absence of the ligand (see reviews, Refs. 4, 5). Furthermore, the HBD contains at least one transactivation function (7) and a nuclear localization signal (12) that are activated on hormone binding. A very conserved region toward the C-terminus was suggested to be necessary for receptor dimerization (Fig. 5). Our results provide evidence that the C-terminal part of the GR is a multifunctional subdomain involved in at least ligand discrimination, transcription activation, and protein-protein interaction. In this region the amino acid sequence flanking a conserved glutamic acid (E773 in rGR) has the potential to form a negatively charged amphipathic α-helix (35; see schemes in Fig. 5, A and B). Replacement of either pair of hydrophobic residues by alanines (CS1 and CS2) abolished transcriptional activation in the presence of Dex. We could show that this failure is due to severely reduced affinity for the agonist (Fig. 3) and conclude that this region is involved in ligand binding.

The proline and lysine residues (PK-box) next to that putative helical structure are conserved only in the subclass of GR/PR/AR/MR. Although located within this multifunctional region (see below), none of the activities described for the HBD seemed to be affected by deletion of the two residues (Fig. 2C, CD). However, when the CD mutation is combined with CS1 substitution, the agonist/antagonist response is partially inverted. With direct ligand binding we could show that the mutants containing the Ala substitutions (CS1, CS2, and CS1/CD) lost or severely reduced the ability to bind Dex but not RU486 (Fig. 3). We conclude that for rGR the hydrophobic residues in the putative α-helix are involved in both Dex binding and in transcription activation. The semiconserved PK-box, however, appears to be involved directly neither in ligand binding nor in transactivation in a direct manner.

The importance of the amphipathic α-helix has been reported for other nuclear receptors. Point mutations in this conserved region revealed that the C-terminus of the thyroid hormone receptor type α (c-*Erb*Aα) and the retinoic acid receptor-α are indispensable for transactivation, interference with AP-1 activity, and ligand-dependent induction of erythroid differentiation (36), in contrast to equivalent mutations in ER, which retain substantial transactivation ability (21). Furthermore, the mutated c-ErbAα acted in a dominant-negative manner, hence differently than the CS mutations reported in this work. Analogous mutations in the ER were reported by another group to generate dominant-negative receptors that retain agonist binding (37). We conclude that the amino acid residues in the vicinity of the conserved glutamic acid near the C-terminal end are crucial for both transactivation and for ligand binding, depending on the receptor. To our knowledge, the ER, retinoic acid receptor, or thyroid hormone receptor mutants have not been systematically tested in the presence of antihormones, and we predict that some of them may also partly invert the response toward known antagonists.

We observed a certain receptor specificity when we tried to transfer carboxy-terminal mutations to other members of the GR/PR/AR/MR subclass. Generation of the double mutant in PR (PR.CS1/CD) and AR (AR.CS1/CD) did not show the expected transactivation in the presence of RU486 (Fig. 2D). Furthermore, we observed that carboxyl-end truncations of GR do not produce the inversion of agonist/antagonist as reported for a PR derivative (UP-1; 19). The heterogeneity of sequence and length of the unrelated amino acids in our truncations (CTs) are expected to play minor roles in these results. We also tested these constructs on artificial promoters containing symmetric glucocorticoid and progesterone response elements (29) in the presence or absence of additional binding sites for unrelated transcription factors (Tallone, T., and S. Rusconi, unpublished data) and found that the response was the same as in the MMTV promoter (data not shown). This indicates that the hyperreactivity of the CS1/CD mutant to RU486 is promoter independent. The lack of transactivation of our PR truncations that terminate receptor sequence within or downstream of the conserved region (PR CT), allows us to narrow down the putative inhibitory function (19) to the region comprised between residues 879–901 in hPR. We conclude that functions exerted by the putative c-terminal α-helix are partly receptor specific, although this region is highly conserved in the nuclear receptor superfamily. Only systematic swap studies of this region will unveil the role of individual residues.

Functional differences between GR and PR/AR may involve residues elsewhere in the receptor. RU486 was shown to promote DNA binding of the receptors *in vivo* (16), a function that may permit at least the default action of the hormone-independent TAF-1. One could expect that both GR and PR should display the partial response to RU486. However, in our system we observe this only in the case of the full-length GR and not in the chimeras that retain the GR TAF-1. This shows that the TAF-1 default hypothesis does not apply to the chimeras. It will certainly be interesting to measure the activity of the chimeras in different cell types. Finally, fusion proteins with the yeast GAL4 DBD abolished the increased responsiveness of the CS1/CD mutant to the antagonist, suggesting that other GR domains may be involved in the increased response to RU486 by the CS1/CD mutant.

For several nuclear receptors, including GR and PR, it was shown that a protease-resistant structure that correlates with receptor activation is induced by ligand binding (18). This strongly suggests that the carboxyl-tail of the receptor has discrete conformations when bound with either agonists or antagonists. Our results are in accordance with these findings. We further suggest that the conserved region flanking the invariable glutamic acid is directly involved in the conformational change. This structural alteration is dependent on ligand binding and may include the putative α-helix as well as the subsequent receptor tail (see Fig. 5C, i). We consider it likely that the proline residue (P780 in rGR) is important for the orientation of the carboxyl-tail, which discriminates transactivation activity depending on the character of bound ligand (compare right and middle drawings in Fig. 5C, i). We propose that this helical structure may provide a part of the binding pocket for the ligand and may participate in the conformational change necessary for tran-

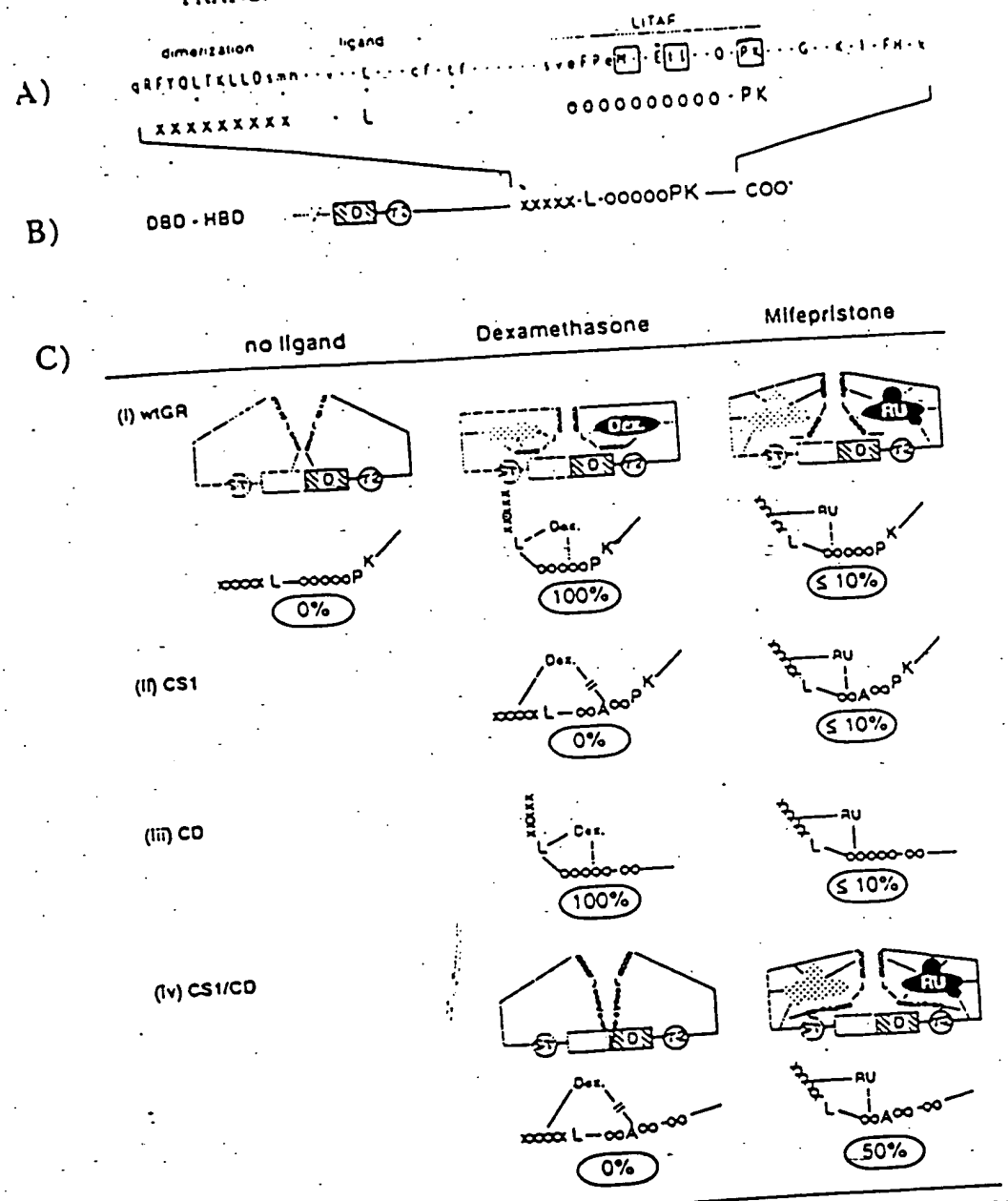


FIG. 5. Model of mutant receptor action. A, Conserved residues in the primary sequence of the carboxyl-end of the GR/PR/AR/MR subclass the nuclear receptor superfamily. Amino acids are shown by the *single-letter code*. *Capital letters*, Identical residues throughout the GR/PR/A MR sub-class; *lower-case letters*, identical residues in at least three members. Potential functions of each section are indicated: *crosses*, t conserved heptad repeat of hydrophobic residues (indicated by *dots*) potentially involved in receptor dimerization (42–44); L, residue reported be crucial for ligand binding (42); *circles*, (subdomain) putative amphipathic α-helix presumably involved in ligand binding, transactivation, a protein-protein interaction (21, 36), termed LITAF; *asterisk*, invariant Glu residue. B, Schematic representation of the DBD-HBD of ster receptors. *Dashed box*, DBD; *circled τ-2*, weak transactivation function described for GR and ER (10); symbols in the carboxy-terminal part described in A. C, Model for mechanism of hormone receptor action. For wtGR and CS1/CD, the model also includes the action of homodimeri receptors. The N-terminal domain is not shown. For all the constructs a schematic interpretation of the C-terminal structure is shown. position of the PK-box is indicated; other symbols are as in A. In this model the structural alteration exerted by ligand binding brings the very terminal tail in a favorable position for transactivation. The substitution of the hydrophobic residues by alanine abolishes Dex binding (ii, L and the C-terminal tail remains in a conformation that is unfavourable for transactivation. RU486 induces a different change, and its bindin not affected by the substitution of the hydrophobic amino acids (ii, *right*). Deletion of the PK-box may alter the structure of the subsequ residues without disturbing transactivation activity, perhaps by partially overcoming the inhibitory function of the carboxyl-tail (iii). combination of both the Ala substitutions and the PK deletion results in a receptor in which the C-terminal part is no longer disturbing after conformational change induced by binding of RU486 (iv, *right*). Dex still cannot bind to this mutant and thus cannot alter the position of receptor tail (iv, *left*).

scription activation and provide a mechanistic interpretation of the effects of mutations (see Fig. 5C, ii–iv) and corresponding legend). In our model we suggest that transactivation of CS1/CD in the presence of the antagonist RU486 requires the interaction of the conserved C-terminal region with the central DBD/*tau*-2 section of the receptor. Since the transactivation activity of *tau*-2 was described for GR (10) but not for PR and AR, one can explain the GR-restricted effects of the CS1/CD mutant observed in our experiments. The available data do not allow us to predict whether the interaction is an intramolecular contact or is mediated by cofactors.

Taken together, we present evidence that sequence conservation does not necessarily imply functional conservation in the GR/PR/AR/MR subfamily. On the basis of our results, we believe that the conserved carboxy-terminal part of GR is a multifunctional region involved in ligand discrimination and determination of agonistic and antagonistic transactivation, and we propose for this subdomain the name LIT [illegible].

## Acknowledgments

We thank Dr. M.-J. Tsai and Dr. A. O. Brinkmann (Erasmus University, Rotterdam, The Netherlands) for the plasmids phPR-*d* and pSV.AR0, respectively, and we are grateful to our colleagues Dr. O. Georgiev and Dr. K. Seipel for the vectors G5-OVEC and pSCTEV, and Roussel-Uclaf for free supply of labeled and unlabeled RU486. We thank Dr. W. Schaffner for generous support, Dr. C. Hovens and M. Koedood for critical reading of the manuscript, and C. Torres for technical assistance.

## References

1. Beato M 1989 Gene regulation by steroid hormones. Cell 56:335–344
2. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
3. Green S, Chambon P 1988 Nuclear receptors enhance our understanding of transcription regulation. Trends Genet 4:309–314
4. O'Malley BW 1990 The steroid receptor superfamily: more excitement predicted for the future. Mol Endocrinol 4:363–369
5. Gronemeyer H 1992 Control of transcription activation by steroid hormone receptors. FASEB J 6:2524–2529
6. Giguere V, Hollenberg SM, Rosenfeld MG, Evans RM 1986 Functional domains of the human glucocorticoid receptor. Cell 46:645–652
7. Webster NJG, Green S, Jin JR, Chambon P 1988 The hormone binding domain of estrogen and glucocorticoid receptors contain an inducible transcription activation function. Cell 54:199–207
8. Tora L, White J, Brou C, Tasset D, Webster N, Scheer E, Chambon P 1989 The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell 59:477–487
9. Godowski PJ, Picard D, Yamamoto KR 1988 Signal transduction and transcriptional regulation by glucocorticoid receptor-LexA fusion proteins. Science 241:812–816
10. Hollenberg SM, Evans RM 1988 Multiple and cooperative transactivation domains of the human glucocorticoid receptor. Cell 55:899–906
11. Brink M, Humbel BM, DeKloet RE, Van Driel R 1992 The unliganded glucocorticoid receptor is localized in the nucleus, not in the cytoplasm. Endocrinology 130:3575–3581
12. Picard D, Yamamoto KR 1987 Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J 6:3333–3340
13. Raynaud J-P, Ojasoo T 1986 The design and use of sex-steroid antagonists. J Steroid Biochem 25:811–833
14. Baulieu EE 1989 Contragestion and other clinical applications of RU486, an antiprogesterone at the receptor. Science 245:1351–1357
15. Busso N, Collart M, Vassalli JD, Belin D 1987 Antagonist effect of RU486 on transcription of glucocorticoid-regulated genes Cell Res 173:425–430
16. Meyer M-E, Pornon A, Ji J, Bocquel M-T, Chambon P, Gronemeyer H 1990 Agonistic and antagonistic activities of RU486 on functions of the human progesterone receptor EMBO J 9:3932
17. Rusconi S, Yamamoto KR 1987 Functional dissection of the hormone and DNA binding activities of the glucocorticoid receptor EMBO J 6:1309–1315
18. Allan GF, Leng X, Tsai SY, Weigel NL, Edwards DP, Tsai MJ, Malley BW 1992 Hormone and antihormone induce distinct conformational changes which are central to steroid receptor activation J Biol Chem 267:19513–19520
19. Vegeto E, Allan GF, Schrader WT, Tsai M-J, McDonnell D, O'Malley BW 1992 The mechanism of RU486 antagonism is dependent on the conformation of the carboxy-terminal tail of human progesterone receptor Cell 69:703–713
20. Lanz RB, Hug M, Gola M, Tallone T, Wieland S, Rusconi S 19 Active, interactive and inactive steroid receptor mutants. Stero 59:148–152
21. Danielian PS, White R, Lees JA, Parker MG 1992 Identifican of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J 11:102 1033
22. Westin G, Gerster T, Müller MM, Schaffner G, Schaffner W 19 OVEC, a versatile system to study transcription in mammalian ce and cell-free extracts. Nucleic Acids Res 15:6787–6798
23. Wieland S, Galli I, Schatt M, Severne Y, Rusconi S 1990 Gene and biochemical analysis of a steroid receptor. In: Sekeris CE, Ale M (eds) Activation of Hormone and Growth Factor Recepto Kluwer Academic Publishers, Dordrecht, pp 215–225
24. Seipel K, Georgiev O, Schaffner W 1992 Different activat domains stimulate transcription from remote ('enhancer') and pro imal ('promoter') positions. EMBO J 11:4961–4968
25. Rusconi S, Severne Y, Georgiev O, Galli I, Wieland S 1990 novel expression assay to study transcriptional activators. Ge 89:211–221
26. Miesfeld R, Rusconi S, Godowski PJ, Maler BA, Okret S, Wik trom AC, Gustafsson JA, Yamamoto KR 1986 Genetic complemen tation of a glucocorticoid receptor deficiency by expression of clone receptor cDNA. Cell 46:389–399
27. Brinkmann AO, Faber PW, van RHC, Kuiper GG, Ris C, Klaasse P, van dKJA, Voorhorst MM, van LJH, Mulder E 1989 The huma androgen receptor: domain structure, genomic organization an regulation of expression. J Steroid Biochem 34:307–310
28. Sadowski I, Ptashne M 1989 A vector for expressing GAL4(1–147 fusions in mammalian cells. Nucleic Acids Res 17:7539
29. Severne Y, Wieland S, Schaffner W, Rusconi S 1988 Metal bindin 'finger' structures in the glucocorticoid receptor defined by site directed mutagenesis. EMBO J 7:2503–2508
30. Elsen LP, Reichman ME, Thompson EB, Gametchu B, Harrison RW, Elsen HJ 1985 Monoclonal antibody to the rat glucocorticoic receptor. Relationship between the immunoreactive and DNA-binding domain. J Biol Chem 260:11805–11810
31. Misrahi M, Atger M, d'Auriol L, Loosfelt H, Meriel C, Fridlansky F, Guiochon MA, Galibert F, Milgrom E 1987 Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. Biochem Biophys Res Commun 143:740–748
32. Chang CS, Kokontis J, Liao ST 1988 Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. Proc Natl Acad Sci USA 85:7211–7215
33. Ellston JF, Beekman JM, Tsai SY, O' Malley BW, Tsai MJ 199 Hormone activation of baculovirus expressed progesterone receptors. J Biol Chem 267:5193–5198
34. Elsen HJ, Schleenbaker RE, Simons SSJ 1981 Affinity labeling of the rat liver glucocorticoid receptor with dexamethasone 21-mesylate. Identification of covalently labeled receptor by immunochemical methods. J Biol Chem 256:12920–12925
35. Zenke M, Munoz A, Sap J, Vennstrom B, Beug H 1990 v-erbA oncogene activation entails the loss of hormone-dependent regulator activity of c-erbA. Cell 61:1035–1049
36. Saatcioglu F, Bartunek P, Deng T, Zenke M, Karin M 1993 A

conserved C-terminal sequence that is deleted in v-ErbA is essential for the biological activities of c-ErbA (the thyroid hormone receptor). Mol Cell Biol 13:3675–3685
37. Ince BA, Zhuang Y, Wrenn CK, Shapiro DJ, Katzenellenbogen BS 1993 Powerful dominant negative mutants of the human estrogen receptor. J Biol Chem 268:14026–14032
38. Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM 1987 Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237:268–275
39. Hollenberg SM, Weinberger C, Ong ES, Cerelli G, Oro A, Lebo R, Thompson EB, Rosenfeld MG, Evans RM 1985 Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 318:635–641
40. Yamamoto KR 1985 Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet 19:209–252
41. Ma J, Ptashne M 1987 Deletion analysis of GAL4 defines two transcriptional activating segments. Cell 48:847–853
42. Fawell SE, Lees JA, White R, Parker MG 1990 Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. Cell 60:953–962
43. Forman BM, Samuels HH 1990 Interactions among a subfamily of nuclear receptors: the regulatory zipper model. Mol Endocrinol 4:1293–1301
44. Lees JA, Fawell SE, White R, Parker MG 1990 A 22-amino-acid peptide restores DNA-binding activity to dimerization-defective mutants of the estrogen receptor. Mol Cell Biol 10:5529–5531